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Genetics of Resistance to Preharvest Aflatoxin Accumulation in Maize Containing the Lfy Gene.

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Genetics of Resistance to Preharvest Aflatoxin Accumulation in Maize Containing the Lfy Gene

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ABSTRACT

Preharvest infection of maize (Zea mays L.) grain by Aspergillus flavus Link ex Fries and subsequent aflatoxin contamination is a serious problem, especially in the southeastern USA. Studies on the genetics of resistance to aflatoxin contamination in maize are quite limited, but a better understanding of this aspect is needed for developing resistant germplasm. The objective of the first field study was to determine the genetics of resistance to aflatoxin contamination in maize possessing the leafy (Lfy) gene. Seven Lfy synthetic genotypes were crossed in a diallel fashion, and the resulting 21 single crosses were evaluated for aflatoxin contamination in three Louisiana environments. Twenty-one days after mid-silk, ears were slash-inoculated with A. parasiticus Speare. Aflatoxin contamination differed significantly among the three environments. General combining ability mean squares were slightly greater than specific combining ability mean squares for aflatoxins B_1, B_2, G_1, and G_2. Crosses involving genotypes Wf9 and B73 had the lowest aflatoxin concentrations, indicating that these genotypes may have some resistance to aflatoxin contamination. High additive genetic correlations suggested that increasing genetic resistance to one toxin should lead to cross resistance to the other three toxins.

A second field study was undertaken to determine the difference in aflatoxin production by A. flavus and A. parasiticus via silk inoculation. Seven maize synthetics containing the Lfy gene, grown in three environments, were inoculated twice, i.e., 14 and 21 days after mid-silk, by atomizing over silks a 2 ml suspension of conidia containing 20 X 10^6 spores ml^{-1} of either A. flavus or A. parasiticus. Aflatoxin

contamination of maize by A. flavus occurred in all three environments, but contamination by A. parasiticus was detected in samples from only one environment where moisture stress occurred. Aspergillus flavus produced significantly higher levels of aflatoxin B_1 and B_2 than did A. parasiticus, suggesting that A. flavus was a more aggressive invader of maize kernels via silks. Differentiation among genotypes for aflatoxin contamination was not possible with the silk inoculation.

INTRODUCTION

It has been 30 years since the discovery of aflatoxin when 100, 000 turkeys died from ingestion of contaminated peanut (Arachis hypogea L.) meal (Lancaster et al., 1961). Subsequently, scientists have extensively studied aflatoxin contamination of agricultural commodities. Aflatoxins are secondary fungal metabolites produced by Aspergillus flavus Link ex Fries and A. parasiticus Speare (Davis and Diener, 1983). The toxins are carcinogenic to laboratory animals and livestock, and have been linked to liver cancer in humans (Bodine and Mertens, 1983; Pier, 1987). Contamination occurs in several agricultural crops but is of greatest concern in maize (Zea mays L.), peanuts, and cotton (Gossypium hirsutum L.). Since these commodities are used as both feed and food, researchers are continuously looking for ways to eliminate or reduce contamination.

Losses from aflatoxin contamination of maize occur in production, marketing, and utilization processes. Direct costs to farmers take the form of yield losses, non-marketable grain, restricted markets, increased transportation costs and lower market prices, increased cost of drying and selling, and inability to obtain loans on stored grain (Nichols, 1983). In 1980, maize growers in the southeastern USA were estimated to have lost over $97 million (Nichols, 1983).

Host-plant resistance seems to be the most effective way to reduce preharvest aflatoxin contamination. Due to the sporadic nature of fungal infection and variability of aflatoxin levels, consistency and accuracy in field experimentation have been difficult to achieve. Although artificial inoculation methods have led to greater consistency in

field research, these methods may be environment specific. Screening of maize germplasm has been extensive (Kang et al., 1988; Kang et al., 1990; Lillehoj et al., 1975b; Lillehoj et al., 1983a; Lillehoj et al., 1983b; Scott and Zummo, 1990; Scott and Zummo, 1988; Zuber et al., 1978), but no genotype to date has been identified with complete resistance. Researchers have identified varying degrees of aflatoxin resistance in maize inbreds, hybrids, and populations (Scott and Zummo, 1988; Scott and Zummo, 1990; Zuber et at., 1983; Zuber et al., 1978). Studies to elucidate the genetic mechanism of resistance in maize are quite limited. Such studies are essential for breeders to develop proper breeding strategies for increasing resistance. Furthermore, there are two species of Aspergillus that produce aflatoxin and information on host resistance to both species is needed.

There is a great deal of maize germplasm that has not been screened or genetically evaluated for resistance to aflatoxin contamination. Therefore, the objectives of the following studies were 1) to determine the genetics of resistance to preharvest aflatoxin levels in maize with the Lfy gene; 2) to compare aflatoxin production of A. flavus vs. A. parasiticus via silk inoculation; 3) to determine if the silk inoculation is adequate to screen genotypes for aflatoxin resistance in Louisiana.

Literature Review

Aflatoxins

Aflatoxins are carcinogenic compounds produced by two fungal species, A. flavus Link ex Fries and A. parasiticus Speare (Davis and Diener, 1983); these compounds are carcinogenic to many animal species including rats, dogs, turkeys, ducklings, pigs, rainbow trout, and others, and may cause liver damage in man (Irvin, 1987; Lillehoj and Zuber, 1975; Ong, 1975). Aflatoxins have been found in agricultural commodities such as maize (corn), peanuts, cotton seed, wheat (Triticum aestivum L.), rice (Oryza sativa L.), sorghum (Sorghum bicolor L. Moench), and tree nuts. The U.S. Food and Drug Administration has set regulatory levels for aflatoxins in corn, cotton seed, and peanuts in the USA. Initially, aflatoxin contamination was thought to be a postharvest problem due to improper storage of a commodity. However, research indicated that infection of maize by Aspergillus and subsequent aflatoxin contamination also occurred prior to harvest (Lillehoj and Zuber, 1975).

A disease epidemic in England in the early 1960's, which caused the death of 100,000 turkeys, led to the discovery of aflatoxins (Lancaster et al., 1961). Death was attributed to aflatoxin-tainted peanut meal. Aflatoxins are difuaranocoumarins (Shotwell, 1986) and include four closely related toxins: aflatoxins B_1, B_2, G_1, and G_2. Aflatoxin B_1 is the most toxic of the four toxins with G_1, G_2, and B_2 exhibiting progressively decreased toxicity (Ong, 1975). Another toxin, M_1, was found in cows' milk after the animals ingested contaminated grain; the toxin is thought to be a derivative of B_1 (Ong,

1975). Contaminated maize grain generally contains aflatoxin B_1 in the highest quantity of the four toxins.

Aflatoxins are produced as secondary fungal metabolites of the polyketide biosynthetic pathway (Steyn and Vleggaar, 1986). The pathway begins with acetyl-coenzyme A, which undergoes condensation reactions with malonyl-SCoA to form the polyketide and, finally, aflatoxin. Many mycotoxins are produced through the polyketide pathway. Since mycotoxins are secondary metabolites, they are thought not to be necessary for survival of the fungus (Lillehoj, 1983).

Aflatoxin poisoning in livestock (aflatoxicosis) has been well documented (Bodine and Mertens, 1983; Hamilton, 1987; Pier, 1987). The LD_{50} for a number of test animals ranges from 0.3 to 10 mg of toxin/kg of body weight (Lillehoj and Zuber, 1975; Ong, 1975). Susceptibility of animals is influenced by age, sex, and health. There is an increasing concern for the effects of aflatoxin on human health. Studies have linked aflatoxin to liver cancer in humans in certain areas of the world (Irvin, 1987). At the sub-cellular level, aflatoxins bind to DNA, causing modification in RNA synthesis and, in some instances, suppression of transcriptional activity (Pier, 1987) and associated development of hepatomacarcinomas (Ong, 1975; Rosiles, 1987). Effects of aflatoxicosis on livestock include reduced feed consumption, weight loss, low milk yield, reproductive complications, and increased susceptibility to other diseases (Bodine and Mertens, 1983; Hamilton, 1987).

Aspergillus Species

Aspergillus flavus and A. parasiticus are the only two fungi known to produce aflatoxins (Diener and Davis, 1987). These fungi are classified as saprophytes, but they can also be parasitic on some plants and insects (Diener et al., 1987; Lillehoj, 1987; Widstrom, 1979). Aspergillus flavus is generally adapted to aerial and foliar environments and is associated with foliage-feeding insects (Lillehoj et al., 1980c). Aspergillus parasiticus routinely inhabits the soil environment and soil insects. Corn, cotton seed, and tree nuts are generally infected by A. flavus, whereas A. parasiticus generally invades peanuts (Diener et al., 1987). Of the four aflatoxins, A. flavus produces only toxins B_1 and B_2 , whereas A. parasiticus produces aflatoxins B_1, B_2, G_1, and G_2. Furthermore, A. flavus can lose its ability to produce aflatoxin much more quickly than A. parasiticus on laboratory media (Diener and Davis, 1987).

Aspergillus species reproduce asexually, with conidia being produced on conidiophores. Conidiophores of A. parasiticus are generally less than 500 um in length and 400 to 1000 um for A. flavus, with conidiophores of both species having roughened exterior walls (Wicklow, 1983). Both species have small, echinulate conidia, compared with other Aspergillus species, that are produced in single chains from phialides. Colony color for A. flavus is yellow-green and ivy-green for A. parasiticus (Wicklow, 1983). Sclerotia formed from fungal mycelia are the primary overwintering stage for Aspergillus spp. Germination of sclerotia in the spring leads to conidial development. Sclerotia, conidia, and mycelia in plant debris or in insects can serve as primary sources of inoculum (Diener and Davis, 1987). Conidia producted on infected plant parts serve as

secondary sources of inoculum (Diener et al., 1987). Dispersal of conidia generally occurs by air movements, but they can also be dispersed by insects.

Infection of Maize by Aspergillus

One of the earliest reports of infection of maize by Aspergillus was by Taubenhaus (1920) in Texas in 1920. He observed that erect maize ears, which tended to collect water, had the greatest infection. Furthermore, infection was generally linked to damaged kernels. Infection by A. flavus and subsequent aflatoxin contamination have been recognized as a problem not only in the southeastern USA since the mid-1970s, but occasionally in the Corn Belt in certain years (Lillehoj et al., 1980b; Kilman, 1989; Zuber et al., 1976). Prior to the 1970s, contamination of maize by aflatoxin was considered to be a post-harvest problem. However, studies by Lillehoj et al. (1975a) indicated that aflatoxin contamination also occurred prior to harvest. Much of the preharvest infection by A. flavus was thought to occur only after the ears were predisposed to infection by insect feeding damage (Lillehoj et al., 1975a; Lillehoj et al., 1980c; Zuber and Lillehoj, 1979). Rambo et al. (1974) also reported that some type of kernel wounding was needed for invasion by the fungus.

Aspergillus flavus can colonize maize silks and infect undamaged kernels (Jones et al., 1980; Marsh and Payne, 1984). Diener et al. (1987) also reported that natural invasion by Aspergillus species can occur through the silks. There have been several studies on factors that affect the degree of colonization and infection. It seems that optimal colonization occurs in a fairly narrow time-frame, and timing of colonization on

silks is important. In a study using silk color as an indication of optimal colonization, Marsh and Payne (1984) found that colonization and infection were highest when yellow-brown silks were inoculated with A. flavus spores. Young green silks were not favorable for colonization and subsequent infection (Diener et al., 1987). Optimum infection by A. flavus occurred when spores were sprayed on silks 2 to 3 weeks after mid-silk, and dead brown silks were not conducive to colonization (Jones et al., 1980).

Temperature has a pronounced effect on colonization and infection. Jones et al. (1980) observed 73% kernel infection in maize grown at 32 to 38 degrees C and only 7.5% infection at 21 to 26 degrees C when silks were inoculated. Payne et al. (1988) also found increased kernel infection at higher temperatures and indicated that A. flavus might have an increased parasitic ability at higher temperatures. High relative humidity also favored colonization of silks.

Plant stress may facilitate greater colonization of maize kernels and infection by Aspergillus. Jones et al. (1981) noted a higher incidence of infection by A. flavus in non-irrigated corn than in irrigated corn. Other factors such as weed pressure and fertility stress may lead to increased infection (Zuber and Lillehoj, 1979). Increased inoculum levels may lead to greater infection; these levels are influenced by many environmental factors (Payne, 1983).

Invasion of the maize kernel by Aspergillus has been a controversial subject (Payne, 1987). Some investigators believe invasion by the mycelium may occur at the silk scar, whereas others believe invasion occurs at the hilar layer. However, most evidence suggests that the fungus enters the kernel through the hilar layer (Diener et al.,

1987; Fennell et al., 1973; Lee et al., 1980). The stage of kernel development at which the fungus invades has also been studied. Rambo et al. (1974) and Zuber and Lillehoj (1979) reported that kernels are most susceptible to infection in the late-milk to early-dough stage. The degree of kernel infection also may vary with Aspergillus species. Calvert et al. (1978) observed that A. flavus was more aggressive than A. parasiticus in invasion of maize kernels.

Aspergillus Growth and Aflatoxin Production

Factors that affect growth of Aspergillus species and aflatoxin biosynthesis include moisture, temperature, substrate, aeration, and others (Lillehoj, 1983). Many tests identifying pertinent factors have been limited to laboratory experiments. Aflatoxin contamination of maize occurs mainly in the southeastern USA, where high temperatures and relative humidity prevail during crop development (Zuber et al., 1976). In the field, Aspergillus growth and aflatoxin production would be affected by many variables including the ones noted and the plant's genotype. Factors that affect fungal colonization would most likely influence aflatoxin levels found in maize.

High aflatoxin levels often are associated with plant water stress. Extreme drought in 1988 resulted in unusually high aflatoxin levels in maize in the Corn Belt. High aflatoxin levels in maize were linked to drought stress in 1977 in the southeastern USA (McMillian et al., 1978). Other instances of drought stress have been linked to high aflatoxin contamination in maize (Lillehoj, 1983). In addition to drought stress, nitrogen stress contributed to high aflatoxin levels in maize (Payne et al., 1989); plants

receiving no nitrogen fertilization contained approximately 28% more aflatoxin than plants receiving optimal nitrogen. Other factors affecting the plant environment may also lead to increased aflatoxin levels.

Laboratory experiments identifying factors influencing Aspergillus growth and aflatoxin production are numerous, but they may not mimic the field environment. Growth of the fungus and aflatoxin production have been observed on most grain crops that serve as natural substrates (Lillehoj, 1983). On artificial media, Davis and Diener (1968) found fungal growth and aflatoxin production to occur on glucose, ribose, xylose, and glycerol, all of which served as carbon sources. In another study, Davis et al. (1967) observed aflatoxin production to be greatest when Aspergillus was grown on a sucrose containing media with either aspartate, glycine, glutamine, or glutamate was used as a nitrogen source. Furthermore, magnesium, zinc, and iron are essential for high toxin production. Cotty (1988) found that ammonium sulfate used as a nitrogen source in media increased aflatoxin production two-fold over media containing sodium nitrate; the author claimed that the increased toxin production resulted from a decrease in pH due to the ammonium sulfate.

Aspergillus species are aerobic and respond to changes in atmospheric gases. Increasing CO_2 levels above 20% has proven to decrease fungal growth and aflatoxin production (Landers et al., 1967). Oxygen levels below 5% also reduced fungal growth and toxin synthesis. Sanders et al. (1968) also showed that increased CO_2 levels decreased aflatoxin production. The atmospheric environment in stored maize grain may affect aflatoxin synthesis by Aspergillus.

Aspergillus species are classified as mesophilic organisms. The fungus grows best at 36 to 38 degrees C with a range of 6 to 46 degrees C, but it may grow at higher temperatures on natural substrates (Lillehoj, 1987). Diener and Davis (1966) reported maximum aflatoxin production by A. parasiticus at 25 and 30 degrees C on either peanuts or artificial media, whereas A. flavus produced maximal toxin at 25 degrees C. In controlled experiments with maize, Thompson et al. (1983) found that toxin synthesis was maximal at 26/22 degrees C day/night temperature; this study showed the direct role of temperature effect on aflatoxin production.

Probably the most important factor in the growth of any fungal organism is moisture. Spore germination for Aspergillus species is optimal at 95% relative humidity (RH), but it can also occur below 85% RH (Lillehoj, 1983). In mature maize kernels, it was found that A. flavus growth and aflatoxin synthesis were minimal below 85% RH, but significant quantities of toxin were detected at 86-87% RH (Lillehoj, 1983). Stringent requirements for fungal processes are quite common. Diener and Davis (1978) observed maximal aflatoxin production in peanuts at 90 to 95% RH. Sanders et al. (1968) found minimal aflatoxin production in peanuts at 86% RH. In stored maize grain, A. flavus requires at least 17.5% grain moisture for growth (Lopez and Christensen, 1967). Drying corn grain, after harvest, to approximately 13% moisture is one of the most important methods to prevent aflatoxin contamination during storage.

Insects and Aflatoxin Contamination in Maize

Insects have long been recognized to play a major role in aflatoxin contamination

of maize. Since the early 1970s, when preharvest aflatoxin contamination was recognized as a serious problem, many studies have linked insect damage to high aflatoxin levels (Anderson et al., 1975; Fennell et al., 1975; Lillehoj et al., 1975a; Lillehoj et al., 1980b). Anderson et al. (1975) found that most maize samples with the bright greenish-yellow fluorescence, often associated with aflatoxin contamination, also had insect damage. A regional survey in Georgia (McMillian et al., 1978) indicated a correlation coefficient of 0.52 (P=0.01) between insect damage and aflatoxin contamination. Lillehoj et al. (1980a) also noted a close relationship between insect damage and high aflatoxin levels. Widstrom et al. (1975) suggested that damage by insects may predispose maize ears to invasion by A. flavus and subsequent aflatoxin contamination. Fennell et al. (1975) observed that ears damaged by insects had an incidence of 6.3% A. flavus infection on maize kernels, whereas a 2.5% infection incidence occurred on kernels with no insect damage. In a 6-year study in Georgia, McMillian et al. (1985) observed significant, positive correlations between insect damage, A. flavus infection, and aflatoxin concentration.

It has been established that insects serve as vectors for transporting Aspergillus spores. Insects may carry spores externally and/or internally (Widstrom, 1979). Lillehoj et al. (1980c) found A. flavus to be associated with foliage feeding insects, whereas A. parasiticus was associated with soil insects. Guthrie et al. (1982) demonstrated that A. flavus colonized the European corn borer (ECB) (Ostrinia nubilalis) better than A. parasiticus did. Aspergillus flavus is the predominant of the two aflatoxin-producing species in infected maize. The association between A. flavus and foliage-feeding insects seems

logical and may give the species an advantage in infection of preharvest corn over A. parasiticus.

Numerous insects have been identified as contributors to aflatoxin contamination in maize including the maize weevil (Sitophilus zeamais), ECB, fall armyworm (FAW) (Spodoptera frugiperda), corn earworm (CEW) (Heliothis zea), and others. Barry et al. (1985) observed that the maize weevil may serve as a vector for Aspergillus under some conditions, but the wheat curl mite (Eriophyes tulipae) may not. Lillehoj et al. (1982) demonstrated that damage of maize kernels by the ECB resulted in elevated aflatoxin levels. Lillehoj et al. (1984) also found that damage by the CEW led to significantly increased aflatoxin levels. There seem to be mixed results on delineation of those insect species that lead to the highest aflatoxin levels. However, such results may demonstrate the specificity of an insect in a particular environment. Widstrom et al. (1975) observed that ears damaged by the ECB had higher aflatoxin levels than ears damaged by either the FAW or CEW. In contrast, Barry et al. (1986) found that CEW-damaged ears had higher aflatoxin levels than ears damaged by the ECB. Fennell et al. (1975) collected insects from ears infected by A. flavus and found the fungus to be associated with a higher proportion of CEW than ECB. According to McMillian, (1987) the maize weevil increased kernel infection and aflatoxin concentration more than FAW, ECB, or the CEW did.

Insecticides and/or host-genotypes resistant to insects have exhibited some success in reducing aflatoxin contamination. Widstrom (1976) noted that insecticide treatment of maize reduced insect damage and aflatoxin contamination, but it did not eliminate the

problem. Generally, the use of insecticides for control of insects and aflatoxin in maize is not economical. Lillehoj et al. (1984) studied maize hybrids, one resistant and one susceptible to the CEW, for aflatoxin presence. Aflatoxin levels were not significantly different between the hybrids. Barry et al. (1986) found that tight husks, a trait associated with insect resistance, reduced aflatoxin contamination.

Insects inevitably play a role in aflatoxin contamination of maize. Damage by insects is usually erratic from year to year and location to location. To reduce the aflatoxin problem caused by insects, the most desirable solution is to develop and use insect-resistant varieties.

Inoculation Techniques

Screening genotypes for disease resistance is an integral part of most plant breeding programs. Identification of aflatoxin-resistant genotypes has been difficult due to the erratic nature of infection by Aspergillus. Natural infection has not been reliable enough for screening for resistance. Artificial inoculation techniques often result in higher and more uniform levels of infection and aflatoxin contamination, and allow differentiation of genotypes.

Early studies indicated that artificial kernel wounding was necessary to obtain high enough aflatoxin levels for differentiation of genotypes. Rambo et al. (1974) compared silk inoculation (silks sprayed with a spore suspension) with two kernel injury methods. One procedure involved injecting spores into kernels with a syringe and needle and the other was insertion of a spore-impregnated cotton swab into a hole in the ear. No visible

infection resulted from silk inoculations, whereas kernel infection was observed with the injury methods. LaPrade and Manwiller (1976) inoculated six hybrids by 1) using silk inoculation, 2) applying spores to the surface of kernels, 3) injecting inoculum into a single kernel, or 4) injecting inoculum into three kernels at different locations in the ear. Infection and aflatoxin production were found only in injured kernels. Calvert et al. (1978) compared three injury methods for kernel infection in maize lines differing in kernel pericarp thickness. Injury techniques included 1) the pinbar method (sewing pins arranged in rows and mounted on a plastic holder), 2) razor blades mounted in a holder, and 3) the hypodermic syringe and needle. Holes were punctured in kernels with the pinbar, whereas kernels were slit with the razor; conidia were sprayed on kernels in both techniques. Aflatoxin levels were highest when the pinbar and razor blade methods were used. Thick pericarp genotypes had lower aflatoxin levels than did thin pericarp genotypes. Widstrom et al. (1981) also found that elevated kernel damage resulted in higher aflatoxin levels.

Wounding techniques simulate insect damage and may circumvent resistance of the aleurone or pericarp layers to natural infection. Wallin (1986) studied whole kernels and kernels that had been decapped with a razor for selecting for aflatoxin resistance. Decapped kernels had higher aflatoxin levels, suggesting that the pericarp and/or aleurone layers contributed to resistance. To determine genotype resistance to natural fungal invasion, non-injury techniques were needed for screening. Darrah et al. (1987) indicated that a modified natural inoculation (silks sprayed with conidia and covered with plastic and paper bags) would be more desirable than kernel wounding for screening

genotypes. Some success has been achieved via use of non-injury methods for screening genotypes. Jones et al. (1980) obtained significant infection by Aspergillus with the silk inoculation procedure; inoculations were most effective at one and two weeks after mid-silk. Scott and Zummo (1988) compared the pinbar (a plastic bar with a single row of 35 stainless steel pins) method to two non-injury techniques. The pinbar method gave highest infection, but the two non-injury techniques also differentiated hybrids. Zummo and Scott (1989) compared the pinbar method with five non-injury methods. They noted that two non-injury techniques (needle through husk, and needle in silk channel) resulted in adequate infection for screening genotypes. King and Scott (1982) found that two techniques that caused kernel damage, pinbar and injection of spores into a kernel, allowed differentiation between genotypes for resistance to aflatoxin, whereas the two non-injury techniques, silk channel and exposed kernel, were not effective. In other studies, the pinbar method provided the best differentiation of genotypes for resistance to aflatoxin (Tucker et al., 1986; Scott et al., 1991).

Maize Plant Resistance to Aflatoxin

Breeding for disease resistance has been successful in many crops. However, identification of aflatoxin resistance and incorporation of pertinent traits into maize lines has been an enormous challenge for breeders. To develop proper breeding strategies for incorporating resistance to a disease into germplasm, a breeder must 1) identify sources of resistance and 2) determine the genetic control of resistance. Zuber (1977) stated, "Mycotoxin levels in corn could be controlled by inherited differences in the ability to:

1) resist invasion of the fungus into the kernel, 2) minimize amount of fungal growth within a kernel or 3) inhibit mycotoxin synthesis." Breeders have the choice of screening for any of the three denoted differences in the aflatoxin contamination process. However, utilization of artificial inoculation techniques has not eliminated a large degree of variability (high coefficients of variation) in aflatoxin experiments. Testing of genotypes over years and locations is necessary for differentiation of aflatoxin resistance since a large genotype X environment interaction is often encountered (McMillian et al., 1982; Zuber et al., 1983). For aflatoxin field studies, it has been suggested eight replications may be needed for best efficiency, when cost of aflatoxin analysis and reduction in standard error are considered (Gardner et al., 1987).

Genotypic differences for aflatoxin contamination have been observed in some studies. Lillehoj et al. (1976) compared six hybrids at two locations for aflatoxin contamination. Significant differences were observed among genotypes for aflatoxin B_1 levels and the results were consistent across locations. In another study, Lillehoj et al. (1975b) examined normal and opaque-2 endosperm types for aflatoxin levels; there was no significant effect of endosperm type on aflatoxin contamination. In a study of maize genotypes with varied endosperm characteristics, Lillehoj et al. (1983b) reported significant differences among the endosperm types for aflatoxin levels. The cross Mo17 X B73 and a waxy hybrid had the highest aflatoxin levels. McMillian et al. (1982) evaluated popcorn genotypes for aflatoxin resistance; significant differences were reported, but a large genotype X environment interaction also was noted.

Widstrom et al. (1978) studied commercial hybrids and several experimental

three-way crosses. Commercial hybrids differed significantly for aflatoxin levels, whereas the experimental three-way crosses did not. The lack of differences among the latter was attributed to a lack of requisite replications and/or a common single-cross tester. Lillehoj et al. (1983a) compared short-, mid-, and full-season hybrids for aflatoxin levels. The short-season hybrids had significantly higher aflatoxin levels than did either the mid-season or full-season hybrids. The authors stated that elevated grain moisture levels at harvest in early-season hybrids may have resulted in higher toxin levels. In a study of open-pollinated and hybrid varieties, Zuber et al. (1983) reported that an open-pollinated variety, Huffman, had the highest aflatoxin levels, whereas another open-pollinated variety, Yellow Creole, had the least. Kang et al. (1988) screened hybrids in the Louisiana state yield trials for natural field aflatoxin contamination, and significant differences were reported among hybrids in both full-season and medium-early season hybrids. The study was unique in finding aflatoxins B_1, B_2, G_1, and G_2 in naturally-infected maize grain. The results suggested that significant infection by A. parasiticus occurred where A. flavus generally predominates. In another study, Kang et al. (1990) crossed 12 genotypes of broad genetic base with two testers. Significant differences were noted among the genotypes and testers. Identification of aflatoxin resistance in genotypes with broad genetic base would be useful in breeding programs. Scott and Zummo (1988) screened 50 inbreds for kernel infection by Aspergillus. Inbred MP313E had the lowest percent kernel infection (3.6%), whereas SC212M had the highest percent kernel infection (62.1%). Significant differences have also been reported for aflatoxin levels between two populations that were developed from kernels

on a segregating maize ear (Widstrom et al., 1987).

Although studies have reported significant genotypic variation for resistance to aflatoxin, the level of resistance has not been sufficient for commercial introduction. Therefore, breeding strategies to characterize resistance are necessary. Genetic studies to elucidate the gene action controlling resistance have been limited and produced inconsistent results. Results were confounded by the environment, inoculation technique, and kernel sampling procedure. For example, Zuber et al. (1978) conducted the first genetic study in maize on aflatoxin resistance by employing a diallel mating design among eight inbreds; they used the pinboard inoculation technique and bulked all kernels on the ear for aflatoxin analysis. Results showed that the general combining ability (GCA) was significant, whereas specific combining ability (SCA) and reciprocal effects were not, indicating that the genetic control of aflatoxin resistance was additive in nature. Zuber et al. (1978) suggested that aflatoxin production in maize might be reduced by a cyclic selection program. However, since the entire ear was sampled for analysis, variability in ear size of the 28 crosses could have biased the results. Gardner et al. (1987) used a diallel mating among seven of the eight lines used by Zuber et al. (1978). In the study, the pinboard inoculation method was used, and only kernels damaged by the pinboard were assayed for aflatoxins. The results showed that both GCA and SCA were significant. Since SCA accounted for two-thirds of the total genetic variation, the observation underscored the importance of dominance and epistatic effects. However, assay of damaged kernels may not be appropriate since a resistance factor may have been compromised in the kernels. Darrah et al. (1987) studied the same seven inbreds as used

by Gardner et al. (1987) and seven of the eight inbreds used by Zuber et al. (1978) in a diallel crossing scheme. In the study, the modified natural inoculation technique was used in which spores were sprayed onto the silks and then covered with plastic and paper bags. Results of the study showed that the GCA mean square was highly significant and accounted for most of the genetic variation. Darrah et al. (1987) compared results of the three diallel studies and noted inconsistency in estimates of GCA effects between their study and the two studies that used the pinboard inoculation procedure. For example, inbred lines H84 and Mo5 had positive GCA estimates for aflatoxin production when the pinboard method was used, but negative estimates with the modified natural inoculation. Inbred N104 had a negative GCA effect when the pinboard method was used, but a positive estimate when the modified natural inoculation technique was employed. Widstrom et al. (1984) evaluated two sets of maize inbreds, through a diallel mating, for aflatoxin resistance. The researchers inoculated the base, middle, and tip of the ear with a hypodermic syringe 20 days after full silk. One diallel consisted of nine dent inbreds and the other included eight sweet corn inbreds. Data combined over years indicated significant GCA effects, whereas SCA effects were not important in either grain type. In these maize genotypes, additive genetic effects were largely responsible for aflatoxin production.

Aflatoxin production in maize seems to be genetically controlled in a quantitative manner, and like most quantitative traits is influenced by the environment. Inoculation technique and kernel sampling procedure may also influence aflatoxin production. Genetic studies utilizing an expanded maize germplasm base are needed for further

elucidation of resistance.

Aflatoxin Detection in Maize

Initial identification of aflatoxin contamination in maize is often based on a presumptive test (Shotwell, 1983); under a black light (365 nm) aflatoxin-contaminated corn exhibits a bright-greenish yellow fluorescence (BGY). Grain elevators and government agencies often use the black light or presumptive test for aflatoxin detection because it is so easily done. However, the test is not always accurate and further assay is necessary for confirmation. The BGY material is a product of kojic acid, another fungal metabolite, and not aflatoxin, but it is still a good indicator of aflatoxin (Shotwell, 1983). The BGY fluorescing material was first noted in maize in the early 1970's. Fennell et al. (1973) noted a good correlation between BGY and aflatoxin contamination in white corn. They indicated that kernels needed to be coarsely ground to identify any "hidden" BGY. Lillehoj et al. (1983a) also reported a positive and significant relationship between aflatoxin and BGY. Shotwell et al. (1975) found that of 569 samples that contained BGY particles, only 55% contained measurable levels of aflatoxin. The authors noted that the BGY fluorescing test could not be used to quantify aflatoxin. Lillehoj et al. (1980a) pointed out, the "BGY fluorescence is a reasonably accurate qualitative indicator of aflatoxin presence when the corn is contaminated with high levels of aflatoxin." They also found that BGY particles commonly occurred in maize that was not contaminated with toxin. Similarly, Kwolek and Shotwell (1979) stated that the BGY test should not be used as a quantitative predictor of aflatoxin contamination. Shotwell

(1983) described other rapid screening methods for aflatoxin detection including thin-layer chromatography.

To confirm the presence of aflatoxin in maize, quantitative procedures such as thin layer chromatography and high pressure liquid chromatography are used (Shotwell, 1986; Wilson, 1987). According to Shotwell (1983), there are three major steps in aflatoxin analysis including extraction, purification, and quantification of the toxin. The Association of Official Analytical Chemists (AOAC) (1984) published an extensive manual on aflatoxin analyses. The AOAC adopted and recommended the CB method, named after the Contaminants Branch (CB) of the Food and Drug Administration (FDA), for aflatoxin analysis in maize. Presently, the FDA has set a maximum acceptance level of 20 ng g^{-1} of aflatoxin in maize grain used for human consumption.

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Chapter 1

Combining Ability for Resistance to Field Aflatoxin Accumulation in Maize Possessing the Lfy Gene

Abstract

Aflatoxin contamination of maize (Zea mays L.) grain prior to harvest frequently occurs in the southeastern USA. The carcinogenic properties of aflatoxins have caused great concern among both producers and consumers of maize. Genetic studies relative to mechanisms of resistance to aflatoxin production in maize germplasm are limited and non-existent in maize containing the Lfy gene. The objective of this study was to determine general (GCA) and specific (SCA) combining abilities for resistance to preharvest aflatoxin levels in maize possessing the Lfy gene. Twenty-one single crosses from a diallel mating of seven Lfy maize synthetic genotypes were evaluated for aflatoxin contamination in three environments in Louisiana. Twenty-one days after mid-silk, ears were slash-inoculated with Aspergillus parasiticus Speare. Samples were analyzed for aflatoxins B_1, B_2, G_1, and G_2. Significant differences were observed for aflatoxin contamination among the three environments. The GCA mean squares were slightly greater than SCA mean squares, but only values for aflatoxins B_2, G_1, and G_2 were significant. Estimates of GCA effects for genotypes Wf9 and B73 were negative for all four aflatoxins, indicating that these genotypes may be useful in reducing aflatoxin contamination. Additive genetic correlations were relatively high and significant among the four aflatoxins (0.76* to 0.96**) with the exception of the correlation between aflatoxins B_1 and G_2 (0.43). This suggested that increasing resistance to one toxin should lead to cross resistance to the other three toxins.

Introduction

Infection of maize grain by Aspergillus flavus and subsequent aflatoxin contamination prior to harvest are serious problems, especially in the southeastern USA. For over a decade, researchers have been diligently screening diverse maize germplasm for resistance to aflatoxin accumulation (Kang et al., 1988; Kang et al., 1990; Lillehoj et al., 1976; Scott and Zummo, 1988; Widstrom et al. 1978; Zuber et al., 1983; Zuber et al., 1978). Due to the erratic nature of fungal infection and aflatoxin production, obtaining consistent results across environments on host resistance has been difficult (Widstrom and Zuber, 1983; Zuber, 1977). To date, there are no known genotypes with immunity to aflatoxin accumulation, but varying degrees of genetic resistance have been identified (Scott and Zummo, 1988; Scott et al., 1991). Genetic studies are an extremely important aspect of aflatoxin research since such studies can help breeders identify mechanisms of resistance and develop proper breeding strategies for incorporating resistance into useful breeding material. Only a few genetic studies have been conducted with respect to aflatoxin resistance.

Most genetic studies have indicated that additive genetic effects are more important in determining aflatoxin resistance than non-additive effects. Zuber et al. (1978) conducted the first genetic study which involved an eight-line diallel. They sampled whole ears for aflatoxin contamination and found general combining ability (GCA) to be more important than specific combining ability (SCA). Gardner et al. (1987) used seven of the same eight lines and inoculation method (pinboard) as used by Zuber et al. (1978), but only sampled injured kernels. In this study, SCA accounted for

a greater amount of the sums of squares than did GCA. However, damage to the kernels may have precluded any resistance in the pericarp and aleurone layers. Darrah et al. (1987) used the modified silk inoculation on a diallel cross involving the same seven lines as used by Gardner et al. (1987). Combined over five environments, GCA was more important than SCA. The studies by Zuber et al. (1978) and Gardner et al. (1987) each utilized only a single location. Since aflatoxin production varies greatly with environment, it is important to test genetic material in several environments. Widstrom et al. (1984) evaluated nine dent and eight sweet corn lines in separate diallel matings in several environments. They also reported GCA to be more important than SCA. In addition to the environment, inoculation and/or sampling methods may influence aflatoxin production on maize grain.

Germplasm evaluated in inheritance studies is quite limited. Three of the four studies noted above used the same genetic material. Therefore, it is desirable that genetics of resistance to aflatoxin accumulation be studied in new maize germplasm, especially that containing the Lfy gene (Shaver, 1983). Factors such as plant stress, drought stress, and insect damage have been associated with aflatoxin contamination (Zuber and Lillehoj, 1979; Widstrom, 1979). Genotypes with greater tolerance to these factors may also possess resistance to aflatoxin. Shaver (1983) reported that maize genotypes that contain the Lfy gene may have greater drought tolerance and insect resistance.

Therefore, we wanted to test the hypothesis that the Lfy gene material may show resistance to aflatoxin accumulation. The objective of the current study was to determine

general and specific combining abilities for resistance to aflatoxin accumulation via a diallel mating among seven maize genotypes containing the Lfy gene.

Materials and Methods

Seven synthetic maize genotypes containing the Lfy gene were crossed in a diallel fashion to produce 21 possible single crosses. The Lfy gene has been patented (Shaver, 1983). The seven synthetics, A619, A632, B73, HY, Wf9, Mo17, and 914, were developed by Cornnuts, Inc., Oakland, CA and provided to M. S. Kang, under licensing, as subscription material. The 21 single crosses were grown at the Ben Hur Plant Science Farm, Baton Rouge, LA in 1988 and 1990 and at the Northeast Research Station (Macon Ridge Branch), Winnsboro, LA in 1990. Seed of each genotype were planted into a Commerce silt loam (Aeric Fluvaquent, fine-silty, mixed, nonacid, thermic) on 13 April, 1988 and 26 March, 1990 at Baton Rouge and into a Gigger silt loam (Typic Fragiudalf, fine-silty, mixed, thermic) on 22 March, 1990 at Winnsboro. The experiments were conducted utilizing a randomized, complete-block design with four replications at Baton Rouge in 1988 and three replications at both locations in 1990. The single crosses were grown in one-row plots of 6.1 m length with 102 cm between rows and a 30 cm within row spacing. Plots at Baton Rouge were fertilized with 200 lb $acre^{-1}$ N, 104 lb $acre^{-1}$ P_2O_5, and 104 lb $acre^{-1}$ K_20 in both years. Plots at Winnsboro, LA were fertilized with 120 lb $acre^{-1}$ N, 80 lb $acre^{-1}$ P_2O_5, and 80 lb $acre^{-1}$ K_2O. Weeds were controlled with recommended herbicides. Insect damage to plants and ears was not observed in any environment, therefore insecticides were not applied. Rainfall was quite adequate at Baton Rouge in both years so irrigation was not neccessary. Plots were irrigated at Winnsboro with 3.5 cm water at silking.

Fifteen ears per plot were inoculated 21 days after mid-silk (50% plants with

silks). Inoculations were made using the slash technique (a modification of knife inoculation method developed at Baton Rouge by M. S. Kang) (Scott et al., 1991). This technique involves dipping a knife into a conidial spore suspension of 20 X 10^6 spores/ml of Aspergillus parasiticus (SRRC 2999), cutting through the husks, and injuring one kernel row. Due to some bird damage and low aflatoxin levels in the 1988 test, the technique was slightly modified for the 1990 test. In 1990, a knife was used to cut through the husks and injure one kernel row, then 1 ml of fungal spore suspension was atomized over the injured kernel row and a rubber band was placed around the ear to secure the husks. In all environments, ears were hand-harvested five weeks after inoculation and dried for three days at 60°C. Inoculated ears were hand-shelled by removing one row of kernels on either side of the knife-damaged row. A 50 g sub-sample of grain from each plot, not inlcuding knife-damaged kernels, was ground for aflatoxin quantification. Samples were stored at 4°C at all times when not in use to inhibit fungal growth and/or aflatoxin production. Assays for aflatoxins B_1, B_2, G_1, and G_2 were done at the USDA Southern Regional Research Center, New Orleans, LA using thin-layer chromatography and densitometry (Association of Official Analytical Chemists, 1984).

Logarithmic (base 10) transformations of aflatoxin concentration (ng g^{-1} + 1) were used to stabilize the variance (Snedecor and Cochran, 1967). Geometric means (Snedecor and Cochran, 1967) were obtained by taking the antilog of the logarithmic means. Data were combined over environments and subjected to the analysis of variance (SAS, 1985). The data was analyzed using Griffing's (1956) diallel method 4, model 1

(genotypes as fixed effects). Genetic correlations of GCA effects among aflatoxins B_1, B_2, G_1, and G_2 were calculated.

Results and Discussion

Aflatoxin contamination of maize grain was observed in all three environments (Table 1.1). Aflatoxin B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2, respectively) concentrations were significantly higher at Winnsboro in 1990 than at Baton Rouge in either year. Corn grown at the Baton Rouge in 1988 had the lowest aflatoxin levels. The modification of the slash inoculation technique in 1990 apparently led to higher levels of aflatoxin production. Plant stress is often associated with high aflatoxin levels (Zuber and Lillehoj, 1979). Extreme water stress occurred during grain filling at Winnsboro and this factor may be partially responsible for the highest aflatoxin levels at that location. Favorable growing conditions for maize existed at Baton Rouge in 1988, where aflatoxin concentrations were lower.

Overall mean aflatoxin levels were highest for AFB_1, followed by AFG_1, AFB_2, and AFG_2 (Table 1.1). Aflatoxin B_1 is generally found in the highest concentration of the four toxins in contaminated maize. However, since AFG_1 occurred at relatively high levels in the 1990 tests, it may be advantageous to screen genotypes for resistance to Aspergillus parasiticus because Aspergillus flavus produces only AFB_1 and AFB_2, whereas A. parasiticus produces all four toxins (Davis and Diener, 1983). The argument for using A. parasiticus is strengthened by recent investigations by Scott and Zummo (1990), who found that genotypes resistant to A. flavus were also resistant to A. parasiticus.

The coefficients of variation ranged from 32% for AFB_1 to 58% for AFG_2 (Table 1.1). Although these values are quite high, they are lower than those reported in

previous genetic studies (Darrah et al, 1987; Gardner et al, 1987; Zuber et al, 1978). The lower CV's may suggest that the slash inoculation procedure used in this study provided more consistent aflatoxin levels than did other techniques.

In the combined analysis of variance, mean squares for environments were highly significant for all four aflatoxins (Table 1.2). The GCA mean squares were significant for AFB_2, AFG_1, and AFG_2, but not for AFB_1 (Table 1.2). Only the SCA mean square for AFG_1 was significant. The GCA and SCA mean squares for AFB_1 and AFG_1 were of the same magnitude, but GCA mean squares for AFB_2 and AFG_2 were about 1.5 times greater than the corresponding SCA mean squares. Since the GCA mean squares were, at least, slightly larger than SCA mean squares, additive, additive x additive, and higher order interaction of additive genetic effects were considered to be more responsible for aflatoxin production in these synthetics than non-additive effects. However, non-additive genetic effects also contributed to the control of aflatoxin production, especially for AFB_1 and AFG_1. Genetic studies by Darrah et al. (1987), Widstrom et al. (1984), and Zuber et al. (1978) revealed that GCA was much more important than SCA. However, a study by Gardner et al. (1987) found that SCA accounted for most of the total genetic variation. Interpretation of these studies would suggest that different genetic systems in a genotype may govern aflatoxin resistance separately depending on the germplasm, environmental conditions, inoculation procedure, and the aflatoxin measured.

The environment x cross interaction effect was significant for AFG_2 only (Table 1.2). The partitioning of this interaction revealed that the environment x GCA effect was significant and largely responsible for the environment x cross interaction for AFG_2.

Since AFG_2 generally occurs at extremely low levels, this interaction is of little consequence. The nonsignificant environment x cross interaction effects for AFB_1, AFB_2, and AFG_1 indicated relative consistency for contamination of crosses over the three environments (Table 1.2). Environment x SCA mean squares were not significant for any aflatoxin.

Genotypes Wf9 and B73 had negative GCA effects for all four aflatoxins, but only values for AFG_1 were significantly different from zero (Table 1.3). This suggested that these two genotypes tended to lower aflatoxin accumulation. A632 had negative GCA effects for three of the aflatoxins whereas, the other four genotypes tended to increase aflatoxin contamination. Genotypes A619, HY, and Mo17 had positive GCA effects for all toxins, but these values were not significantly different from zero, with the exception of the value for HY for AFB_1 (Table 1.3). The changes in sign of the GCA values for genotypes A632 and 914 across aflatoxins might indicate that different genetic systems within a genotype controlled aflatoxin production of different aflatoxins. The cross A619 x 914 had the only significant, positive SCA effects for all four aflatoxins (data not shown), indicating that this cross was more susceptible than other crosses. There were no other single crosses that had significant and positive or negative SCA effects for all toxins. No single cross from this material with Lfy gene showed immunity to aflatoxin contamination.

Genotypes Wf9 and B73 ranked 1 and 2, respectively, for having the lowest GCA values for all four toxins (Table 1.3), indicating that resistance factors in these two genotypes must be operating against all four aflatoxins. Ranks of GCA effects for A619

and HY were relatively high but not consistent across the four aflatoxins.

Correlations among GCA effects for different traits are additive genetic correlations (Griffing, 1956). These correlations are due to pleiotropic effects of genes and not due to linkage (Griffing, 1956). Genetic correlations among GCA effects for all aflatoxins were significant with the exception of the correlation of AFB_1 with AFG_2 (Table 1.4). The implication here is that there are genes that have a pleiotropic effect and condition resistance/susceptibility to all four aflatoxins simultaneously. Selection for resistance to one of the toxins should increase resistance to the other three toxins.

We concluded that aflatoxin production in the seven synthetics was under genetic control and was significantly influenced by the environment. The Lfy synthetic genotypes Wf9 and B73 tended to depress levels of all four aflatoxins. Since GCA was slightly greater than SCA for all aflatoxins, a recurrent selection program to concentrate favorable alleles for resistance might be effective. However, the relatively large experimental error in relation to the amount of genetic variability would suggest that progress would be slow. This study provided useful information on sources and inheritance of resistance in maize possessing the Lfy gene.

Table 1.1. Geometric† means of concentrations of four aflatoxins for a seven maize parent diallel grown in three environments in Louisiana.

Environment	Year	AFB_1	AFB_2	AFG_1	AFG_2
		----------ng g^{-1}----------			
Northeast Station (Winnsboro)	1990	426.5‡A	49.7 A	339.7 A	32.3 A
Ben Hur (Baton Rouge)	1990	117.4 B	11.4 B	53.7 B	5.7 B
Ben Hur (Baton Rouge)	1988	45.6 C	4.3 C	2.9 C	1.4 C
Mean		196.5	21.8	132.1	13.1
CV (%)		32	52	43	58

†Antilogarithm of the logarithmic mean.

‡Means within a colunm followed by the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

AFB_1=Aflatoxin B_1, AFB_2=Aflatoxin B_2, AFG_1=Aflatoxin G_1, AFG_2=Aflatoxin G_2.

Table 1.2. Analysis of variance for concentration of four aflatoxins in maize crosses among seven Lfy synthetics according to Griffing (1956).

Source	df	AFB_1	AFB_2	AFG_1	AFG_2
			Mean squares		
Environments (E)	2	16.75**	20.24**	79.32**	32.36**
Replications:E	7	0.43	0.69*	0.39	0.19
Crosses (C)	20	0.46	0.28	0.49	0.26
GCA	6	0.65	0.85*	0.83*	0.46*
SCA	14	0.62	0.52	0.78*	0.30
E X C	40	0.43	0.43	0.45	0.35*
E X GCA	12	0.38	0.53	0.46	0.52**
E X SCA	28	0.45	0.39	0.44	0.27
Error	140	0.44	0.33	0.38	0.21

*, ** Significant at the 0.05 and 0.01 probability levels, respectively.

AFB_1=Aflatoxin B_1, AFB_2=Aflatoxin B_2, AFG_1=Aflatoxin G_1, AFG_2=Aflatoxin G_2.

Table 1.3. Mean estimates of general combining ability effects and their ranks (in parenthesis) for concentration of four aflatoxins produced in seven maize genotypes containing the Lfy gene in three environments.

Genotype	AFB_1	AFB_2	AFG_1	AFG_2
A619	0.058 (6)	0.058 (7)	0.098 (6)	0.024 (5)
A632	-0.018 (4)	-0.007 (3)	0.015 (4)	-0.037 (3)
B73	-0.064 (2)	-0.072 (2)	-0.142*(2)	-0.045 (2)
HY	0.183*(7)	0.057 (6)	0.102 (7)	0.010 (4)
Mo17	0.001 (5)	0.038 (5)	0.006 (3)	0.045 (6)
Wf9	-0.123 (1)	-0.108 (1)	-0.149*(1)	-0.054 (1)
914	-0.037 (3)	0.034 (4)	0.069 (5)	0.056 (7)

* Significantly different from zero at the 0.05 level of probability.

AFB_1=Aflatoxin B_1, AFB_2=Aflatoxin B_2, AFG_1=Aflatoxin G_1, AFG_2=Aflatoxin G_2.

Table 1.4. Additive genetic correlations of GCA effects for concentrations of aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) in crosses among maize synthetics possessing the Lfy gene.

	AFB_2	AFG_1	AFG_2
AFB_1	0.79*	0.78*	0.43
AFB_2		0.96**	0.85**
AFG_1			0.76*

*,** Significant at the 0.05 and 0.01 probability levels, respectively.

AFB_1=Aflatoxin B_1, AFB_2=Aflatoxin B_2, AFG_1=Aflatoxin G_1, AFG_2=Aflatoxin G_2.

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Chapter 2

Aflatoxin Production by Aspergillus flavus vs. A. parasiticus on Corn Kernels via Silk Inoculation

Abstract

Aflatoxin contamination of corn (Zea mays L.) grain continues to be a serious problem in the southeastern USA. Two Aspergillus species are known to produce aflatoxin. Therefore, information on host resistance to both species is needed. The main objective of this research was to compare aflatoxin production by A. flavus vs. A. parasiticus via silk inoculation. The effect of silk inoculation with A. flavus and A. parasiticus on aflatoxin contamination in seven corn synthetics containing the Lfy gene was studied in three environments in Louisiana. Inoculations were done twice, i.e., 14 and 21 days after mid-silk, by atomizing over external silks a 2 ml suspension of conidia containing 20 X 10^6 spores ml^{-1} of either A. flavus or A. parasiticus. There was no significant differences among environments for aflatoxins B_1 or B_2. Aflatoxin production by A. flavus was detected in corn samples from all three environments, but contamination by A. parasiticus only occurred in samples from Winnsboro, LA. Infection by A. parasiticus may be more environment-specific than that by A. flavus. Aspergillus flavus produced 4.5 times more aflatoxin B_1 and 2 times more aflatoxin B_2 than did A. parasiticus. This indicated that A. flavus was a more aggressive invader of corn kernels via silk inoculation. Silk inoculation did not result in high enough aflatoxin levels to differentiate among genotypes.

Introduction

Aflatoxins are secondary fungal metabolites produced by Aspergillus flavus Link ex Fries and A. parasiticus Speare (5). The carcinogenic properties of aflatoxin have led to great concern since the toxin can occur in both feed and food products. Corn (Zea mays L.) growers, especially in the southeastern USA, are continuously concerned with aflatoxin contamination (21). Contaminated grain may be non-marketable or sold at a reduced price (11). Furthermore, health complications and/or death of farm animals that ingest contaminated grain has been documented (3,9).

Aflatoxin contamination was once thought to be a problem only in stored grain. Studies in the early 1970s (1,10) demonstrated that aflatoxin was also produced prior to harvest. Since then, much research has been directed toward screening genotypes for resistance to aflatoxin accumulation on corn kernels. However, the identification of inoculation techniques that differentiate among genotypes for resistance has been difficult. Techniques that damage kernels to allow quick fungal entry have been most promising in developing high levels of aflatoxin (4,8,16,19,20). Inoculation techniques that do not damage kernels, e.g. silk inoculation, are, generally, more desirable in screening host resistance to aflatoxins for several reasons: 1) they simulate natural infection, 2) they are easy to apply, 3) one can obtain larger kernel sample, and 4) they do not preclude any resistance factor that may be lost when kernel-damaging techniques are used. The silk inoculation has been successful in some experiments (5,13) but not in others (14,16). This indicates that infection via silk inoculation is probably environment-specific. Timing of silk inoculation is also important (7).

Researchers also are interested in finding out whether corn genotypes resistant to A. flavus are also resistant to A. parasiticus. Very limited data are available on this aspect. Calvert et al. (4) determined that both species invade corn kernels, but infection by A. parasiticus was less than that by A. flavus. Recently, Scott and Zummo (15) reported that resistant genotypes were resistant to both species, and susceptible genotypes were susceptible to both species.

Additional information is needed on inoculation techniques and host resistance to both Aspergillus species. Therefore, our primary objective was to determine the difference, if any, in aflatoxin production by A. flavus and A. parasiticus via silk inoculation. We also wanted to determine if silk inoculation would result in adequate aflatoxin levels in corn in Louisiana to differentiate among genotypes.

Materials and Methods

Field experiments were conducted at two locations, Ben Hur and Perkins Road Research Farms in Baton Rouge, LA in 1989, and at Northeast Research Station (Macon Ridge), Winnsboro, LA in 1990. Seven synthetic corn genotypes containing the Lfy gene, used in this study, were: A619, A632, Mo17, B73, HY, Wf9, and 914. This material was obtained from Cornnuts, Inc., Oakland, California. Seed of each genotype were planted into a Commerce silt loam (Aeric Fluvaquent, fine-silty, mixed, nonacid, thermic) at the Ben Hur Farm on April 13, 1989 and into an Olivier silt loam (Aquic Fragiudalf, fine-silty, mixed, thermic) at the Perkins Road Farm on April 4, 1989. In 1990, seed were planted into a Gigger silt loam (Typic Fragiudalf, fine-silty, mixed, thermic) on March 22 at the Northeast Research Station. The tests were planted as a 2 X 7 factorial in a randomized complete-block design with three replications at each location. Plots consisted of single rows of 6.1 m length, with 102 cm spacing between rows and 30 cm spacing between plants. Plots at Ben Hur and Perkins Road Farm were fertilized with 200 lb acre^{-1} N, 104 lb acre^{-1} P_2O_5, and 104 lb acre^{-1} K_2O. Plots at Winnsboro were fertilized with 120 lb acre^{-1} N, 80 lb acre^{-1} P_2O_5, and 80 lb acre^{-1} K_2O. Weeds were controlled with recommended herbicides. Insect damage to plants and ears was not observed in any environment, therefore insecticides were not applied. Rainfall was quite adequate at Ben Hur and the Perkins Road Farm so irrigation was not necessary. Plots at Winnsboro were irrigated with 3.5 cm of water at silking.

The primary ear of each plant was inoculated 14 days and again 21 days after mid-silk (50% plants with silks) at all locations. The second inoculation was done to

insure infection. Inoculations were carried out by atomizing a 2 ml suspension of 20 X 10^6 spores ml^{-1} on external silks. The two treatments were accomplished by applying a spore suspension of conidia of either Aspergillus flavus (SRRC 3357) or A. parasiticus (SRRC 2999). Ears were harvested 5 weeks after the last inoculation date and dried for 3 days at 60°C. Whole ears were machine-shelled and bulked. A subsample of grain from each plot was ground for aflatoxin quantification. Samples were stored at 4°C at all times when not in use to inhibit fungal growth and/or aflatoxin production. Assays for aflatoxins B_1, B_2, G_1, and G_2 were done using thin-layer chromatography and densitometry (2) at the USDA-ARS Southern Regional Research Center, New Orleans, LA.

Logarithmic (base 10) transformations of aflatoxin concentration (ng g^{-1} + 1) were used to stabilize the variance (17). Antilog of the logarithmic means was taken to obtain geometric means (17). Data were combined over environments and subjected to analysis of variance (18).

Results and Discussion

Treatment mean squares were significant for aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2, respectively) (Table 2.1). Aspergillus flavus produces only AFB_1 and AFB_2, whereas A. parasiticus produces AFB_1, AFB_2, AFG_1, and AFG_2. There were significant environment and treatment X environment interaction effects for AFG_1 and AFG_2. This indicated an inconsistency of infection by A. parasiticus and/or production of AFG_1 and AFG_2 across environments.

Aflatoxin levels, averaged over genotypes and treatments for the three environments, are shown in Table 2.2. There were no significant differences among environments for AFB_1 or AFB_2. No AFG_1 or AFG_2 was detected at the Ben Hur Farm or Perkins Road Farm, but a significant amount was detected in corn samples from the Northeast Research Station (Table 2.2). This suggested that A. parasiticus did not infect corn kernels at Ben Hur or Perkins Road. Extremely low levels of AFB_1 and AFB_2 were detected in some plots inoculated with A. parasiticus at those two farms, but since no AFG_1 or AFG_2 was detected, some natural infection by A. flavus must have occurred in plots inoculated with A. parasiticus.

Mean aflatoxin production by A. flavus and A. parasiticus is shown in Table 2.3. Combined over environments and genotypes, A. flavus produced significantly higher AFB_1 and AFB_2 levels than did A. parasiticus. Aspergillus flavus produced over 4.5 times as much AFB_1 and 2 times as much AFB_2 as A. parasiticus. Aspergillus flavus is the predominant of the two species found in contaminated corn grain; although, occasionally, A. parasiticus is also found. Calvert et al. (4) found A. flavus to be more

aggressive than A. parasiticus in invading corn. Extreme water stress occurred at Winnsboro but not at the other locations. Aflatoxin production by A. parasiticus was detected at Winnsboro only. Infection of corn by Aspergillus spp. and high aflatoxin levels are often associated with plant stress (21). This stress may have allowed A. parasiticus to invade kernels more readily via silks at Winnsboro. Results indicated that infection and aflatoxin production by A. parasiticus may be more environment dependent than infection by A. flavus. This dependency may explain, in part, why A. flavus is the predominant of the two species found in infected corn. The higher aflatoxin levels produced by A. flavus indicated that screening genotypes for resistance with A. flavus will allow maximum differentiation among genotypes to be obtained.

Mean AFB_1 levels, in kernels of seven synthetic genotypes, ranged from 11.45 to 2.56 ng g^{-1} (Table 2.4). Genotype A619 had a significantly higher AFB_1 level than Mo17. Aflatoxin B_1 levels of other genotypes were not significantly different from each other or from A619 and Mo17. There were no significant differences among genotypes for AFB_2, AFG_1, or AFG_2. A non-significant genotype X treatment interaction effect (Table 2.1) would indicate that genotypes should be either resistant or susceptible to both species. However, the lack of significant differences among genotypes (Table 2.4) would not allow us to reach this conclusion. Scott and Zummo (15), who used a different inoculation technique and genotypes, did determine that the corn genotypes with resistance were resistant to both species.

The silk inoculation used in this study did not provide high enough aflatoxin levels to differentiate among genotypes. Rambo et al. (14) reported that silk inoculation

was ineffective for obtaining infection and aflatoxin contamination and stated that some type of kernel wounding was needed. However, Jones et al. (7) obtained significant aflatoxin levels, using the silk inoculation. Environment plays a large role in A. flavus infection and aflatoxin production and is probably a key factor responsible for the inconsistencies in experiments where silk inoculation was used. However, the silk inoculation with either species was not effective for screening genotypes in Louisiana.

Table 2.1. Analysis of variance for production of four aflatoxins across seven maize genotypes in three environments.

		Mean squares			
Source	df	AFB_1	AFB_2	AFG_1	AFG_2
Environments (E)	2	0.16	0.12	1.00**	0.07*
Replications:E	6	1.14	0.24	0.43**	0.04
Genotypes (G)	6	0.74	0.15	0.04	0.02
Treatments (T)	1	14.30**	3.19**	1.00**	0.07*
G X E	12	0.46	0.13	0.04	0.02
G X T	6	0.83	0.18	0.04	0.02
T X E	2	2.29	0.14	1.00**	0.07*
E X T X G	12	0.44	0.16	0.04	0.02
Error	78	0.93	0.29	0.12	0.03

*, ** Significant at the 0.10 and 0.01 Probability levels, respectively.

AFB_1=Aflatoxin B_1, AFB_2=Aflatoxin B_2, AFG_1=Aflatoxin G_1, AFG_2=Aflatoxin G_2.

Table 2.2. Geometric† means of concentrations (ng g^{-1}) of four aflatoxins produced by Aspergillus flavus and A. parasiticus over seven maize genotypes in three environments in Louisiana.

Environment	Year	AFB_1	AFB_2	AFG_1	AFG_2
Ben Hur (Baton Rouge)	1989	5.74‡A	1.48 A	0.0 B	0.0 B
Perkins Road (Baton Rouge)	1989	4.40 A	1.75 A	0.0 B	0.0 B
Northeast Research Station (Winnsboro)	1990	5.51 A	1.87 A	1.85 A	1.18 A

†Antilogarithm of the logarithmic mean.

‡Means followed by the same letter are not significantly different according to LSD (0.05).

AFB_1=Aflatoxin B_1, AFB_2=Aflatoxin B_2, AFG_1=Aflatoxin G_1, AFG_2=Aflatoxin G_2.

Table 2.3. Geometric† means for concentration (ng g^{-1}) of four aflatoxins produced by Aspergillus flavus and A. parasiticus in maize kernels via silk inoculation.

Aspergillus spp.	AFB_1	AFB_2	AFG_1	AFG_2
A. flavus	11.26‡ A	2.44 A	-----	-----
A. parasiticus	2.39 B	1.17 B	1.51	1.12

†Antilogarithm of the logarithmic mean.

‡Means followed by the same letter are not significantly different according to LSD (0.05).

AFB_1=Aflatoxin B_1, AFB_2=Aflatoxin B_2, AFG_1=Aflatoxin G_1, AFG_2=Aflatoxin G_2.

Table 2.4. Geometric† means of concentration (ng g^{-1}) of four aflatoxins produced by Aspergillus flavus and A. parasiticus in kernels of seven maize genotypes containing the Lfy gene.

Genotype	AFB_1	AFB_2	AFG_1	AFG_2
A619	11.45‡ A	2.04 A	1.34 A	1.17 A
914	6.76 A B	1.99 A	1.34 A	1.18 A
B73	5.89 A B	1.69 A	0.0 A	0.0 A
HY	4.73 A B	1.68 A	1.20 A	0.0 A
A632	4.34 A B	1.79 A	1.14 A	0.9 A
Wf9	4.19 A B	1.75 A	1.23 A	1.06 A
Mo17	2.56 B	1.09 A	1.37 A	0.0 A

†Antilogarithm of the logarithmic mean.

‡Means followed by the same letter are not significantly different according to LSD (0.05).

AFB_1=Aflatoxin B_1, AFB_2=Aflatoxin B_2, AFG_1=Aflatoxin G_1, AFG_2=Aflatoxin G_2.

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GENERAL CONCLUSIONS

A field study was undertaken to determine the genetics of resistance to preharvest aflatoxin accumulation in seven maize synthetics possessing the Lfy gene. There was a significant influence of the environment on aflatoxin contamination of the 21 single crosses. The increased water stress at Winnsboro partially explained the highest aflatoxin levels there. General combining ability (GCA) mean squares were slightly greater than specific combining ability mean squares, indicating that additive genetic effects controlled aflatoxin accumulation in these synthetics more than non-additive genetic effects. Estimates of GCA effects for genotypes Wf9 and B73 were negative. Therefore, progeny from crosses with these genotypes would tend to have, on average, lower aflatoxin contamination. No single cross from this material showed immunity to aflatoxin contamination. Additive genetic correlations were relatively high among the four toxins, with the exception of the correlation between aflatoxins B_1 and G_2. Increasing resistance to one toxin should lead to cross resistance to the other toxins.

In a second field study, aflatoxin production on maize grain by Aspergillus flavus and A. parasiticus via silk inoculation was compared. Aflatoxin production by A. flavus was observed in all three test environments, whereas aflatoxin production by A. parasiticus was detected in only one environment (Winnsboro, 1990). This suggested that infection by A. parasiticus maybe more environment-specific. Aflatoxin B_1 and B_2 production by A. flavus was significantly greater than that of A. parasiticus. Aspergillus flavus was a more aggressive invader of maize kernels via silk inoculation. However, the silk inoculation did not result in adequate aflatoxin levels to differentiate genotypes.

APPENDICES

Appendix 1. Geometric† means for concentrations (ng g^{-1}) of four aflatoxins for 21 single crosses of maize containing the Lfy gene grown at Ben Hur Farm in 1988.

Cross	AFB1	AFB2	AFG1	AFG2
A619 X HY	501.2	31.6	15.8	2.5
A632 X A619	31.6	4.0	2.3	0.0
A632 X HY	50.1	3.5	1.8	0.0
B73 X A619	15.1	1.9	1.5	1.3
B73 X A632	79.4	2.6	3.5	0.0
HY X B73	72.4	2.7	2.2	0.0
Mo17 X A619	19.9	3.4	1.9	0.0
Mo17 X A632	199.5	15.8	5.5	1.8
Mo17 X B73	21.4	2.2	0.0	0.0
Mo17 X HY	39.8	5.8	1.5	0.0
Mo17 X Wf9	100.0	10.1	12.6	5.1
Wf9 X A619	39.8	2.5	0.0	0.0
Wf9 X A632	12.3	0.0	0.0	0.0
Wf9 X B73	15.8	2.2	1.5	0.0
Wf9 X HY	63.1	7.4	3.0	1.5
A619 X 914	125.9	8.7	12.5	3.7
A632 X 914	15.8	3.4	7.6	2.2
B73 X 914	44.7	3.4	0.0	0.0
HY X 914	173.8	12.5	9.5	1.7
Mo17 X 914	39.8	4.4	12.0	4.3
Wf9 X 914	10.9	1.9	0.0	0.0

† Antilogarithm of the logarithmic mean.
AFB1 = Aflatoxin B_1, AFB2 = Aflatoxin B_2, AFG1 = Aflatoxin G_1, AFG2 = Aflatoxin G_2.

Appendix 2. Geometric† means for concentrations (ng g^{-1}) of four aflatoxins for 21 single crosses of maize containing the Lfy gene grown at Ben Hur Farm in 1990.

Cross	AFB1	AFB2	AFG1	AFG2
A619 X HY	19.9	1.9	22.9	1.4
A632 X A619	147.9	15.8	79.4	8.3
A632 X HY	204.1	13.8	69.2	12.6
B73 X A619	63.1	5.1	33.1	2.5
B73 X A632	181.9	15.8	77.6	6.2
HY X B73	199.5	19.1	100.0	12.5
Mo17 X A619	112.2	8.9	45.7	3.4
Mo17 X A632	125.8	22.9	72.4	19.1
Mo17 X B73	83.2	7.8	28.2	3.6
Mo17 X HY	288.4	19.5	141.3	8.7
Mo17 X Wf9	204.2	20.9	102.3	11.7
Wf9 X A619	128.8	10.0	43.7	3.0
Wf9 X A632	134.9	12.0	63.1	5.0
Wf9 X B73	109.6	11.7	31.6	6.5
Wf9 X HY	125.9	8.1	36.3	2.2
A619 X 914	741.3	70.8	371.5	34.7
A632 X 914	346.7	32.4	218.7	14.5
B73 X 914	47.8	7.8	17.4	5.2
HY X 914	72.4	8.3	33.9	3.5
Mo17 X 914	87.1	9.9	34.7	6.0
Wf9 X 914	16.9	2.9	7.8	1.6

† Antilogarithm of the logarithmic mean.
AFB1 = Aflatoxin B_1, AFB2 = Aflatoxin B_2, AFG1 = Aflatoxin G_1, AFG2 = Aflatoxin G_2.

Appendix 3. Geometric† means for concentrations (ng g^{-1}) of four aflatoxins for 21 single crosses of maize containing the Lfy gene grown at Winnsboro, LA in 1990.

Cross	AFB1	AFB2	AFG1	AFG2
A619 X HY	630.9	12.6	588.8	45.7
A632 X A619	263.0	30.9	316.2	14.1
A632 X HY	446.7	67.6	416.9	38.0
B73 X A619	575.4	107.2	478.6	51.3
B73 X A632	489.8	95.5	426.6	45.7
HY X B73	676.1	104.7	676.2	60.3
Mo17 X A619	616.6	107.2	457.1	37.2
Mo17 X A632	208.9	26.9	158.5	15.1
Mo17 X B73	263.0	31.6	173.8	20.9
Mo17 X HY	537.0	39.8	512.9	60.2
Mo17 X Wf9	223.9	16.9	100.0	16.2
Wf9 X A619	676.1	141.2	588.8	69.2
Wf9 X A632	331.1	41.7	295.1	31.6
Wf9 X B73	602.5	107.2	575.4	81.3
Wf9 X HY	354.8	38.0	281.8	29.5
A619 X 914	602.6	107.2	575.4	81.3
A632 X 914	141.2	16.9	56.2	2.4
B73 X 914	537.0	79.5	489.8	58.9
HY X 914	489.8	44.7	416.9	40.7
Mo17 X 914	371.5	48.9	275.4	21.9
Wf9 X 914	575.4	49.0	537.0	37.2

† Antilogarithm of the logarithmic mean.
AFB1 = Aflatoxin B_1, AFB2 = Aflatoxin B_2, AFG1 = Aflatoxin G_1, AFG2 = Aflatoxin G_2.

VITA

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Agronomy

Title of Dissertation: Genetics of Resistance to Preharvest Aflatoxin Accumulation in Maize Containing the Lfy Gene

Approved:

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